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Evaluation of pretreatment methods for enzymatic saccharification of wheat straw for bioethanol production

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ABSTRACT

Pretreatment is an essential step in the enzymatic hydrolysis of biomass and subsequent production of bioethanol. The current study is focused on two different pretreatment methods of wheat straw using mild temperatures (100 °C for 2 h and RT for overnight). In one method, native substrate was treated with 1.5% (w/v) NaOH at two different above mentioned conditions followed by acid hydrolysis (0.75% (v/v) sulfuric acid at 100 °C for 2 h). In another method, the native substrate was initially treated with acid (0.75% (v/v) sulfuric acid at 100 °C for 2 h) followed by treatment with 1.5% (w/v) NaOH at two different above conditions. After the pretreatments, the residues were treated with Accellerase 1500 (26 U/g) and maximum yield of glucose (65.2 g L^{-1}) were found with 0.75% sulfuric acid (100 °C for 2 h) followed by alkali (1.5% NaOH at 100 °C for 2 h). Fermentation of this hydrolyzate using *Saccharomyces cerevisiae* strain produced 24.4 g L^{-1} of ethanol with corresponding yield of 0.44 g/g.

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1. Introduction

Ethanol from renewable resources has been of interest in recent decades as an alternative fuel to the current fossil fuels. Its production has gained importance in the last few years due to the increased dependency on oil and conventional fuels (Ramanathan, 2000). At present ethanol is produced from molasses. The cost of production increases as the demand for molasses has increased. Hence, it is necessary to search for alternate source for ethanol production. Lignocellulosic biomasses like wood and agricultural crops are abundantly available having rich source of sugars e.g., straw and sugar beet pulp which are potential raw materials for producing several high-value products like fuel ethanol and biodiesel. Up to 80% of the lignocelluloses are polysaccharide. The cost of ethanol production from lignocellulosic materials is relatively high based on current technologies, and the main challenges are the low yield and high cost of the hydrolysis process. Considerable research efforts have been made to improve the hydrolysis of lignocellulosic materials (Sun & Cheng, 2002). The current research investigates the use of acid and enzymes to saccharify lignocellulosic materials and to produce glucose from pretreated lignocellulosic materials, to be a source for ethanol production.

Lignocellulosic material consists of mainly three different types of polymers, namely cellulose, hemicellulose and lignin, which are associated with each other. Wheat straw is the most attractive low cost feedstock for production of fuel alcohol because of abundance, renewability and low lignin content. It has a huge potential for bioethanol production. Wheat straw is an abundant by-product from wheat production, which is annually generated worldwide (529 million tons/year). Asia is the first largest producing region with 43% of global wheat production. The average yield of wheat straw is 1.3–1.4 kg per kg of wheat grain. Wheat straw has to be pretreated before enzymatic hydrolysis since it contains lignin and hemicellulose that protect the cellulose. Research has been done on the separation of cellulose, hemicellulose and lignin components from wheat straw and structural characterization of the hemicellulose fraction (Sun & Cheng, 2002) and also the production of ethanol from wheat straw hydrolyzates (Klinke, Olsson, Thomsen, & Ahrin, 2003; Nigam, 2001).

Pre-treatment disrupts the naturally resistant carbohydrate–lignin shield that limits the accessibility of enzymes or chemicals to the cellulose and hemicellulose. The main goal of pretreatment is to increase the enzyme accessibility and improving digestibility of cellulose (Mosier et al., 2005). Each pretreatment has a specific effect on the cellulose, hemicellulose and lignin fraction thus, different pretreatment methods and conditions should be chosen according to the process configuration selected for the subsequent hydrolysis (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010).

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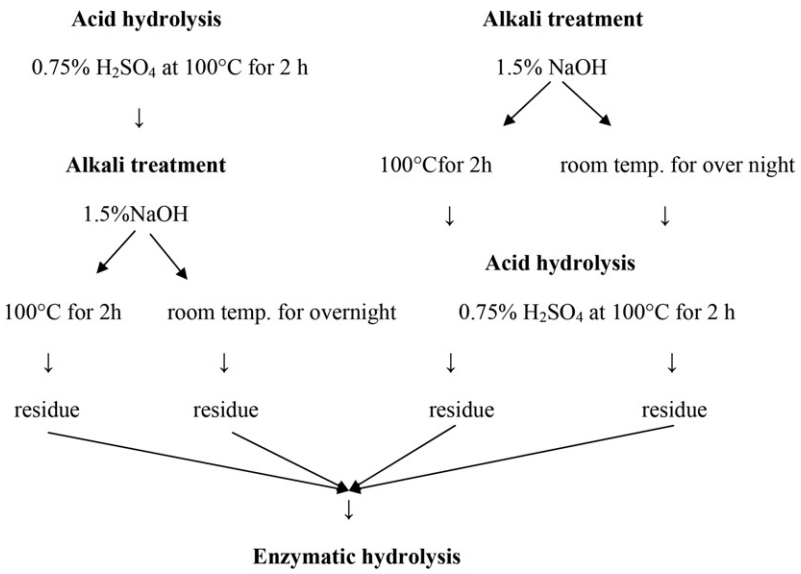

Fig. 1. Flow chart for pretreatment by acid followed by alkali and alkali followed by acid at different conditions using wheat straw.

Acid hydrolysis removes the hemicellulosic portion and some fraction of lignin the remainder of the lignin remains intact to the cellulosic substrate (Kaya, Heitmann, & Thomas, 2000). During enzymatic hydrolysis of lignocellulosic biomass, cellulase components, β-glucosidase and endoglucanase have more binding affinity towards lignin than to the carbohydrates, resulting in a lower efficiency of saccharification. Hence, to achieve maximum hydrolysis of cellulosics, which is a prerequisite for ethanol fermentation, an appropriate delignification treatment of biomass is required (Rishi, Krishna Kant, & Kuhad, 2009).

Alkali treatment disrupts the cell wall through dissolving hemicellulose, lignin, silica, and hydrolyzing uronic and acetic acid esters. Alkali swells cellulose, decreasing the crystallinity of cellulose (Sun, Lawther, & Banks, 1995). All of the ester-linked substituents of the hemicellulose and other cell wall components can be cleaved by alkali (Ternrud, 1987).

Enzymatic hydrolysis of cellulose to glucose is carried out by cellulase enzymes which are highly specific catalysts. The conversion of native cellulose to sugar can be achieved by applying different pretreatment methods namely chemical, acid and enzyme (Waleed et al., 2011). It is necessary to increase the rate of hydrolysis of cellulose to fermentable sugars. Enzyme hydrolysis is performed under mild conditions (e.g., pH 4.5–5.0 and temperature 40–50 °C) and it is possible to obtain cellulose hydrolysis close to 100% (Ogier, Ballerini, Leygue, Rigal, & Pourquie, 1999). Therefore one may expect low corrosion problems, low utility consumption, and low toxicity of the hydrolyzates which is the main advantage of this process (Lee, Iyer, & Torget, 1999; Taherzadeh, 1999). Addition of surfactants during hydrolysis can modify the cellulose surface properties. Among the different surfactants, fatty acid esters of sorbitan polyethoxylates (Tween® 20 and 80) and polyethylene glycol are reported as most effective for enzymatic hydrolysis (Kim, Kim, & Kim, 2006; Börjesson, Peterson, & Tjerneld, 2007).

Several species of bacteria and fungi are able to produce cellulases and hemicellulases (Sun & Cheng, 2002). Fungi like *Trichoderma reesei* or *Trichoderma viride* have been the most broadly studied and best characterized and the best vehicles for cellulase production (Tengborg, Galbe, & Zacchi, 2001; Xia & Shen, 2004). A full complement production of cellulase, stability under the enzymatic hydrolysis conditions, and resistance of the enzyme to chemical inhibitors are the advantages of the cellulase produced by *Trichoderma* (Hari Krishna, Janardhan Reddy, & Chowdary, 2001; Itoh, Wada, Honda, Kuwahara, & Watanabe, 2003). Fuel ethanol production from plant biomass hydrolyzates by *Saccharomyces cerevisiae* is of great economic and environmental significance. The demands on the microorganisms that perform this reaction are more complicated than those for conventional ethanol production from hexoses or their disaccharides, which uses exclusively *Saccharomyces* yeasts (Klinke et al., 2003).

In the present study, effect of pretreatment conditions with alkali, acid and enzymatic saccharification for high sugar yield from wheat straw was examined (Fig. 1). The work reported here is on the investigation of the order of pretreatment method for higher saccharification and also subsequent fermentation of sugars to ethanol.

2. Materials and methods

2.1. Raw material

Wheat straw was collected from Aligarh, Uttar Pradesh, India. The straw was dried and cut into 1–3 cm fiber in a laboratory pulverizer followed by sieving to make the straw dust free and then dried at 60 ± 0.5 °C for overnight. All the chemicals used were of analytical or reagent grade and all the experiments were performed in triplicates and the results are presented as mean ± standard deviation.

2.2. Microorganism

The organism *S. cerevisiae*, identified as VS3 strain in our lab (Kiransree, Sridhar, Suresh, Banat, & Venkateswar, 2000) was used for fermentation studies. Stock cultures of *S. cerevisiae* were maintained and grown on YEPD agar. (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L; and agar, 25 g/L, pH: 5.0 ± 0.2. Stock cultures were stored at 4 °C.)

2.2.1. Inoculum media for organism

Medium used for the inoculum preparation for ethanol fermentation contained (g/L): yeast extract, 10; peptone, 20; dextrose, 20, pH: 5.00 ± 0.5 for 24 h at 28 ± 0.5 °C and 150 rpm (Pasha, Valli, & Rao, 2007).

2.3. Fermentation media

The hydrolyzate was taken along with the supplementation of (g/L): yeast extract, 1.5; $(NH_4)2SO_4$, 1; K_2HPO_4, 0.5; peptone, 1;

Table 1
Chemical composition of wheat straw.

S. no.	Component	% Dry weight
1	Moisture	8 ± 0.3
2	Alcohol extractives	2.5 ± 0.2
3	Hot water extractives	5.5 ± 0.2
4	Acid soluble lignin	16 ± 1.1
5	Acid insoluble lignin	4.6 ± 0.2
6	Cellulose	32.6 ± 0.3
7	Hemicellulose	24.7 ± 0.2

$MgSO_4 \cdot 7H_2O$, 0.5, and $MnSO_4$, 0.5, pH adjusted to 5.5. Inoculum preparation and fermentation were carried out as described by Pasha, Kuhad, and Rao (2007).

2.4. Chemical compositional analysis of wheat straw

The cellulose, lignin and hemicellulosic fractions of pulverized wheat straw were determined according to ASTM (2007) method. The straw was analyzed in duplicate for moisture, hot water extractives, alcoholic extractives, acid soluble lignin, acid insoluble lignin, holocellulose and ash according to the above method. The composition values are tabulated (Table 1).

2.4.1. Procedure

1 g of wheat straw was taken and dried in an oven at 100–150 °C until the constant weight of the substrate was obtained. The percentage of the moisture free sample was calculated. The moisture free substrate was extracted with ethanol–toluene solution for 4 h and the solvent was removed by suction as possible and washed by 50 ml of ethanol to remove the toluene. Excess ethanol was removed and the substrate was transferred to a beaker to be digested with 400 ml hot water in water bath at 100 °C for 3 h. The substrate was washed with 100 ml hot distilled water and 50 ml ethanol after hot water treatment. Residual substrate was dried in the air. To the air dried substrate 15 ml of cold (12–15 °C) 72% H_2SO_4 was added slowly with stirring and allowed to stand for 2 h with frequent stirring at 18–20 °C. After that the material was washed into a 1 L beaker, diluted to a 3% concentration of H_2SO_4 by adding 560 ml of distilled water and boiling for 4 h. The mixture in the beaker was allowed to settle insoluble material, filtered into a crucible (dried at 100–105 °C) and weighed. The residue was washed free of acid with 500 ml of hot water and the contents in crucible were dried in oven for 2 h at 100–105 °C, the drying and weighing was repeated until the weight is constant. Holocellulose content of wheat straw was determined as Wise, Murphy, and D'Addieco (1946).

2.5. Pretreatment

The pretreatment of wheat straw was done in two ways. In the first method wheat straw was treated initially with 1.5% (w/v) NaOH at room temperature (27 ± 2 °C) for overnight and at 100 °C for 2 h separately, followed by 0.75% (v/v) sulfuric acid hydrolysis. The alkali pretreatment was carried out by taking two sets of 25 g dried native wheat straw at 10% substrate level (1:10 ratio) in 500 ml flasks separately; the flasks were treated with 3.75 g NaOH. One flask was placed at room temperature i.e. (27 ± 2 °C), overnight and other at 100 °C for 2 h. The residual substrates were washed and neutralized with water and then dried at 50 °C for further acid hydrolysis. The alkali treated substrate was subjected to acid hydrolysis at 10% substrate level with 0.75% (v/v) sulfuric acid at 100 °C for 2 h.

In the second method 50 g of dried wheat straw was treated with 500 ml of 0.75% (v/v) sulfuric acid in boiling water bath maintaining at 100 °C for 2 h. After acid hydrolysis, the residual substrates were neutralized, dried and differentiated into two equal parts then subjected to alkali treatment with 1.5% (w/v) NaOH at room temperature (27 ± 2 °C) for overnight and at 100 °C for 2 h separately at 10% level.

The contents were squeezed using cheese cloth and the biomass was repeatedly washed with tap water until the pH became neutral. The filtrate was used to estimate the amount of reducing sugars by DNS method. The residual substrates were dried at 50 °C to constant weight and later subjected to enzyme hydrolysis.

2.6. Enzymatic hydrolysis

The residual substrates obtained after the two different treatments were subjected to enzymatic hydrolysis with commercial cellulase, Accellerase 1500 (52 IU/ml). The loading of enzyme was 26 IU per gram of substrate taken for hydrolysis and the hydrolysis was performed under mild conditions (pH 5.0, 150 rpm and temperature 50 °C) in the ratio of 1:20 (0.5 ml enzyme + 19.5 ml acetate buffer pH 5) (w/v) (substrate:enzyme) and incubated for 48 h. The samples were collected at regular intervals of 0 h, 18 h and 24 h.

2.7. Ethanol fermentation

The enzymatic hydrolyzate with the highest sugar concentration was used for ethanol fermentation using our thermotolerant yeast (VS3). The hydrolyzate was supplemented with 1 g/L of yeast extract, peptone, ammonium sulfate, K_2HPO_4 and 0.5 g/L of magnesium and manganese sulfates (Pasha, Kuhad, et al., 2007) and sterilized at 10 psi for 20 min. After cooling the media to 30 °C, VS3 was transferred aseptically at 10% inoculum level. Fermentation was carried out at agitation of 150 rpm for 48 h at the temperature of 30 ± 0.5 °C. Samples were collected at 12 h intervals throughout the fermentation. The ethanol concentration was measured by gas chromatography and the remaining sugars during fermentation were estimated. For calculation of ethanol yield the following equation was applied (Nutawan, Phattayawadee, Pattranit, & Mohammad Naghi Eshtiaghi, 2010):

$$\text{Ethanol yield} = \frac{\text{Measured ethanol in sample (g)}}{\text{Theoretical ethanol (g)}}$$

$$\text{Theoretical ethanol (g)} = \text{amount of initial sugar content (g)} \text{ in fermentation solution} \times 0.5$$

2.8. Analytical methods

2.8.1. Total reducing sugars

The total reducing sugars, present in wheat straw delignified filtrate, acid and enzyme hydrolyzates were estimated by the dinitrosalicylic acid method (Miller, 1959).

2.8.2. Ethanol

Ethanol produced was analyzed by gas chromatography (GC) (Shimadzu 2010, Japan) using ZB Wax column (30 mm × 0.25 mm) with a flame ionization detector (FID).The analysis was performed according to NREL (National Renewable Energy Laboratory) procedure LAP #001.The column temperature was 150 °C (isothermal), program run time: 5.5 min, ethanol retention time: about 2.3 min and the carrier gas was nitrogen (16 kPa), injector temperature: 175 °C, detector temperature: 250 °C, flow rate: 40 ml/min, spilt ratio: 1/50, velocity of H_2 flow: 60 ml/min, sample quantity: 1 μl. One part of the supernatant was filtered by 0.22 μm cellulose acetate filters for GC analysis (Srilekha Yadav, Shaik Naseeruddin, Sai Prashanthi, Lanka Sateesh, & Venkateswar Rao, 2011).

Table 2
Percentage of delignification and saccharification in alkali followed by acid pretreatment.

Treatment	Percentage of delignification	Percentage of sugar loss	Sugars (g/L)	Percentage of saccharification
Pretreated with 1.5% NaOH at RT for overnight	77 ± 1.7	0.9 ± 0.26	0.83 ± 0.04	–
0.75% acid hydrolysate	–	–	10.4 ± 0.55	11.9 ± 0.62
Pretreated with 1.5% NaOH at 100 °C for 2 h	70 ± 1.0	0.9 ± 0.36	0.83 ± 0.09	–
0.75% acid hydrolyzate	–	–	9.8 ± 0.15	12.4 ± 0.26

Table 3
Percentage of delignification and saccharification in acid followed by alkali pretreatment.

Treatment		Percentage of delignification	Percentage of sugar loss	Sugars (g/L)	Percentage of saccharification
Acid treatment	0.75% acid hydrolyzate	–	–	19.6 ± 0.2	20.8 ± 0.25
Alkali treatment	Delignified with 1.5% NaOH at RT for over night	82.7 ± 0.3	1.5 ± 0.03	1.23 ± 0.02	–
	Delignified with 1.5% NaOH at 100 °C for 2 h	79.3 ± 0.32	0.88 ± 0.02	0.76 ± 0.03	–

3. Results and discussion

We could achieve nearly 70% and 77% of lignin loss in the treatment of 1.5% sodium hydroxide at 100 °C for 2 h and room temperature for overnight respectively. The percentages of sugar loss were 0.9% and 0.96%. According to Taherzadeh and Karimi (2007) the efficient delignifier should remove a maximum of lignin and minimum of sugars (not more than 5%). The percentage of lignin loss achieved in the present work is similar to the percentage reported by Cheng (1993) who stated that about 60–70% of wheat straw lignin was removed when treated with 1.5% NaOH at 100 °C for 1 h. Runcang Sun, Mark Lawther, and Banks (2000) reported an optimal condition for delignification was with 1.5% sodium hydroxide for 144 h at 20 °C, resulted in release of 60% of lignin. Dilute acid pretreatment and enzymatic saccharification were evaluated for conversion of wheat straw cellulose and hemicellulose to monomeric sugars. The reducing sugars in the dilute acid hydrolysis after two different alkali treatments were 9.8 g L^{-1} and 10.4 g L^{-1} (Table 2).

Dilute acid hydrolysis is also based on the cleavage of ether bonds by acids between hemicellulose and lignin complexes. In the second method, when the acid hydrolysis has done with 0.75% sulfuric acid at 100 °C for 2 h, 19.6 g L^{-1} reducing sugars were produced. Similar conditions were used by Grohmann, Torget, and Himmel (1985) and Saha, Iten, Cotta, and Wu (2005) in which 0.75% H_2SO_4 at 120–180 °C has solubilized the hemicellulose to fermentable sugars. 79.3% and 82.7% of lignin was removed in two subsequent alkali treatments and nearly 1% sugars were lost (Table 3).

The neutralized substrates were oven dried at 45 °C and further subjected to enzyme hydrolysis with Accellerase 1500 commercial cellulase enzyme (26 U/ml) in the ratio of 1:20 (w/v) (substrate:enzyme) and incubated at 50 °C for 48 h. Sun and Cheng (2002) has reported that, the increase in temperature from 37 °C to 50 °C improved the hydrolysis of cellulose in the enzymatic treatment. This could be explained by an increase of enzymatic activities which have an optimal temperature close to 50 °C. We used Tween 80 (0.1%) as a surfactant in the enzyme hydrolysis. Hydrolysis using enzyme increased the release of glucose by 45.6 g L^{-1} at 24 h. It was found that about 92 per cent of cellulose in the pretreated wheat straw residue which was initially treated with sulfuric acid 0.75% (v/v) at 100 °C for 2 h and then alkali at 100 °C for 2 h converted to glucose by cellulase enzyme. The same result was found in the work carried by Azzam (1989), where 95 per cent of cellulose in the pretreated bagasse pulp residue was converted to glucose by cellulase enzyme. Hence, in the present study the substrate was evaluated for maximum production of ethanol by adopting acid and alkali pretreatment methods followed by fermentation. The saccharification in the other pretreatment methods like; wheat straw first pretreated with sulfuric acid followed by NaOH at room temperature for overnight was 32 g L^{-1}, the substrate treated with 1.5% NaOH at 100 °C for 2 h followed by sulfuric acid treatment was 34 g L^{-1} and the substrate treated with 1.5% NaOH at room temperature (30 ± 2 °C) for overnight followed by sulfuric acid treatment was 30 g L^{-1}. The glucose yields in each interval of enzymatic hydrolysis (0 h, 18 h and 24 h) were shown in Fig. 2.

Only one hydrolyzate which has had the maximum glucose (65.2 g L^{-1}) after enzymatic hydrolysis (45.6 g L^{-1}) was mixed with its acid hydrolyzate (19.6 g L^{-1}) and inoculated with the *S. cerevisiae* (VS3) at 10% (v/v) inoculum level and incubated at 30 °C for 48 h. *S. cerevisiae* was able to utilize 55.2 g L^{-1} of glucose and produced maximum ethanol of 24.4 g L^{-1} at 36 h of incubation with a yield 0.44 g/g and productivity of 0.6 g/L/h (Fig. 3). Recent studies conducted at our laboratory showed ethanol yield 0.431 g/g and productivity 0.5 g/L/h from the mixed hydrolyzate (acidic + enzymatic) of *Lantana camara* fermenting with the same strain (Pasha, Valli, et al., 2007). Martin, Galbe, Wahlbom, Hahn-Hagerdal, and Jonsson (2002) reported ethanol yield (0.38 g/g) from sugarcane bagasse enzymatic hydrolyzate fermenting with *S. cerevisiae* TMB 3001. However, the ethanol production was slightly declined after 36 h of incubation. The ethanol efficiency was found to be 87%. The residual reducing sugars were decreased significantly due to the efficiency

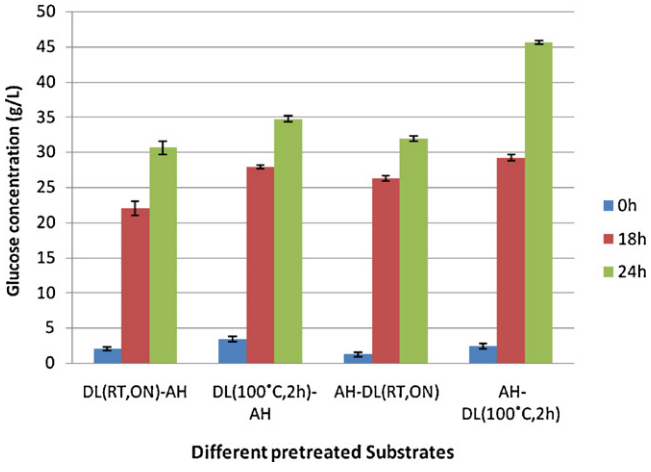

Fig. 2. Enzymatic hydrolysis at 0 h, 18 h and 24 h.

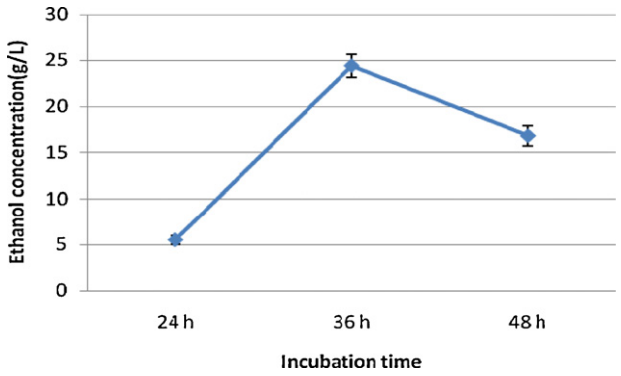

Fig. 3. Ethanol production using *Saccharomyces cerevisiae*.

of the strain which showed the effect of fermentation time on conversion of sugar to ethanol in acid, alkali pretreated and enzyme treated wheat straw. During three days of fermentation period nearly all the fermentable sugars were converted to bioethanol.

4. Conclusion

Pretreatment of wheat straw primarily done with 0.75% sulfuric acid at 100 °C for 2 h then 1.5% NaOH at 100 °C for 2 h was considered as better pretreatment method to get maximum saccharification by enzymatic hydrolysis. When the mixture of both enzyme and acid hydrolyzates was fermented with VS3, it has produced high yield and better efficiency of ethanol.

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